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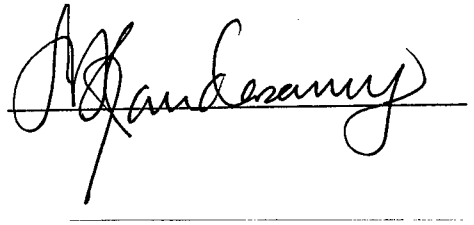

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13. ABSTRACT ***(Maximum 200 Words)***

The overall goal of this research is to understand how the estrogen receptor (ER) signal transduction pathway is altered during breast tumorigenesis and if enhanced and/or aberrant ER signal transduction increases the risk of developing breast cancer. Our previous data suggested that altered expression of ERα, ERβ and their variants occurs during breast tumorigenesis. Our current data suggest that expression of cofactors which enhance ER activity, i.e. SRA, is increased during breast tumorigenesis while expression of cofactors which can repress ER activity, i.e. REA, either does not change or is not increased to a similar extent during breast tumorigenesis. Overall the data support the hypothesis that a substantial alteration of estrogen signaling pathways occurs during human breast tumorigenesis. In addition both our previous and our current data provide a very strong rationale to determine in parallel ER isoform/variant expression and cofactor expression during the early stages of preneoplastic progression that precede the onset of invasive breast cancer and determine if differences exist between normal women and patients who develop breast cancer, in order to assess a potential role in increasing the risk of invasive breast cancer. Identification, retrieval, review and collection of appropriate tissues blocks from a preliminary group of cases/controls in the Manitoba Breast Event Database has been completed and analyses of these tissue sections is in progress.

14. SUBJECT TERMS
Breast Cancer, estrogen receptor, variants, isoforms, cofactors, pre-invasive lesions, preneoplastic lesions, pathology.

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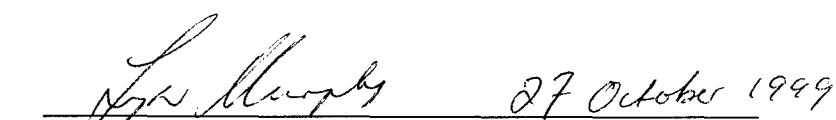

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4. TABLE OF CONTENTS.

5. INTRODUCTION:

The overall goal of this research is to understand how the estrogen receptor signal transduction pathway is altered during breast tumorigenesis and if enhanced and/or aberrant ER signal transduction increases the risk of developing breast cancer.

Current dogma suggests that the steroid hormone, estrogen and its cellular mechanism of action have a major role in both the development and progression of human breast cancer. The estrogen receptor (ER) is a key component in this mechanism which undergoes an apparent increase in level of expression and likely a corresponding increase in signal transduction during tumorigenesis. We and others have compelling evidence to suggest that the expression of the ER, its variants and other isoforms are altered during breast tumorigenesis (1-5). While the expression of specific ER variants and/or isoforms and their overall prevalence relative to wild type ER is different between normal and neoplastic tissue, the pathophysiological significance of these ER variants/isoforms and their potential influence in modulation of the ER pathway in early progression of human breast cancer is not known. We propose to test the hypothesis that increased ER expression and altered ER variant and/or isoform expression cause enhanced and/or aberrant ER signal transduction in breast epithelial cells. We propose that such factors are altered during the early stages of pre-neoplastic progression that precede the onset of invasive breast cancer and are associated with an increased risk of developing breast cancer. Furthermore, in addition to the complexity of the ER-like molecules that may be expressed in target tissues, there is an increasing complexity of factors which can modulate the transcriptional activity of ER which in turn could alter ER signal transduction pathways (6). Recent identification of several of these cofactors (coactivators and corepressors) which can directly modulate the transcriptional activity of ER (7-10), provides compelling rationale to examine their expression in parallel to the studies we have proposed.

Our **specific aim is to determine if alteration of ER signal transduction and ER variant and/or isoform expression occurs during the early stages of pre-neoplastic progression that precede the onset of invasive breast cancer and determine if differences exist between normal women and patients who develop breast cancer.** A case/control retrospective study will be undertaken in which we

will examine ER in comparable breast epithelium and lesions {normal small ducts, benign non-proliferative lesions (adenosis), benign proliferative ductal hyperplasia (PDWA), and ductal carcinoma in situ (DCIS)} in women with or without invasive cancer. ER level will be assessed immunohistochemically, ER-beta mRNA expression will be measured by in situ hybridization, ER function will be assessed by measurement of progesterone receptor and pS2 expression and ER influence on proliferation rate will be assessed by immunohistochemical measurement of Ki-67 in serial paraffin sections. Other factors which have been recently identified to modify ER activity include cyclin D (11), MAP kinase (12), several cofactors such as SRA (9), REA (10), and AIB1 (7). These will be assessed in parallel using antibodies, if available, and *in situ* hybridization. We will also study variant ER mRNA expression in parallel sections from frozen tissue blocks, where possible, by specific RT-PCR assays to detect deleted and truncated ER variants, to determine their relative expression with respect to the wild-type ER mRNAs and assess their potential role in altered ER signaling activity.

6. BODY:

Task 1.

Retrospective study of breast tissue samples. Immunohistochemical and *in situ* hydridization analysis of estrogen receptor signal transduction.

1. We have identified blocks within pathology department archives from one of two selected centers. These blocks contain specific lesions for study and these have now been obtained from St Boniface Hospital as a set of over 300 blocks for this study. A similar retrieval exercise is also underway at a second selected center (The Grace Hospital, Winnipeg) to ensure sufficient cases for the study. Details of these cases are shown on the attached spread sheets (**appendix 1)**. The Manitoba Breast Event Registry (MBED) within the Manitoba Breast Tumor Bank was initially queried for all cases occurring at one center (St Boniface Hospital) over a 1 year period (1997-98). This period was selected to allow 1) sufficient case numbers 2) a wish to select recent cases to ensure ease of retrieval of relatively recent blocks/cases balanced against 3) a wish to ensure that it was unlikely that there were clinical priorities which might necessitate a review of paraffin blocks from any of the cases. This query yielded 149 invasive tumors, 42 in-situ carcinomas, 62 cases with ductal or lobular hyperplasia + other fibrocystic changes, 43 cases with ductal or lobular hyperplasia without other fibrocystic changes, 108 cases with other benign lesions only

including 4 papillomas, 12 fat necrosis, and 43 fibroadenomas, and 59 cases with no reported pathology (examples shown in sheets 'invasive for review', 'in-situ', 'DH,LH,+FCC'). See **appendix 1.**

2. To construct first a series of cases for study comprising normal, benign, proliferative, or in-situ carcinma lesions matched to a synchronous invasive carcinoma in the same patient we have completed a detailed review of all slides from all 149 invasive carcinoma cases. The results are detailed in the attached spreadsheet (invasive reviewed sheet, see **appendix 1**). Specific blocks containing each pathology have now been selected from each case as noted in the appropriate columns (note that the block nomenclature/details vary by individual pathologists practice). The columns are as follows; patient# (MBED patient#), lab# (hospital path lab #), age, agePM (age >=55), invasive, inv+is, is, adh, dh, fcc, n, node met (blocks containing invasive tumor, in-situ components within invasive tumor, in-situ beyond invasive tumor margin, atypical and typical ductal hyperplasia, fibrocystic changes including adenosis and apocrine metaplasia, normal ducts/lobules, nodal metastasis). Invasive tumor type, surgery date, operation type (codes provided in spreadsheet 'MBED codes'). From the initial 149 cases, 79 post-menopausal (>55yrs) cases emerged with invasive tumor and including matching DCIS separate from the invasive tumor in 19 cases, ADH in 10 cases, TDH in 24 cases for example.

We anticipate obtaining a similar number of cases from either the second hospital center and/or extension and review of a more recent second period of 1998-1999 cases from St Boniface hospital.

3. Development and pilot testing of the appropriate controls are in progress. Initially we were to select tumors from the Manitoba Breast Tumorbank representative of negative, low and high levels of appropriate gene expression to use as controls. Since then an additional method has been published documenting the use of cultured cells embedded in agar as a control for quantitative immunohistochemical analysis of estrogen receptor in breast cancer (13). Therefore we are in the process of pilot testing this as an additional control in our study, as well as a control for our *in situ* hydridization analyses.

4. Preliminary studies to determine if certain estrogen receptor cofactors (6), which could modulate ER transcriptional activity, are altered in expression during breast tumorigenesis and/or breast cancer progression *in vivo* have either been completed {SRA, REA, (9, 10) or are in progress (AIB1 (7) p68 helicase (14)} .

We have examined the expression of a novel recently described steroid receptor RNA activator (*SRA*) in human breast tissues. Initially the expression of *SRA* was measured by semi-quantitative reverse-transcription polymerase chain reaction within 27 independent breast tumors, spanning a wide spectrum of grade, estrogen receptor (ER) and progesterone receptor (PR) levels. Subgroup analysis showed that *SRA* expression was similar in ER+/PR+ (median = 65.5, n = 8) and in ER-/PR- (median = 94.6, n = 5) tumors. Interestingly, *SRA* expression in these two subgroups was significantly (Mann-Whitney rank-sum test, $p < 0.05$) lower than that observed in ER+/PR- (median = 156.4, n = 6) and ER-/PR+ (median = 144.8, n = 8) tumors. A variant form of *SRA*, presenting a deletion of 203 bp within the *SRA* core sequence, was also observed in breast tumor tissues. The relative expression of this new *SRA* isoform correlated with tumor grade (Spearman coefficent $r = 0.53$, $n = 27$, $p = 0.004$). These data suggest that changes in the expression of *SRA* related molecules occur during breast tumor progression (15). These data are now published (see **appendix 2).**

As well, using reverse transcription polymerase chain reaction assays, expression of *SRA* was compared between adjacent normal human breast tissue and matched breast tumors from 19 patients. Core *SRA* RNA was detected in normal and neoplastic breast tissues. The level of *SRA* RNA was significantly (Wilcoxon test, $p = 0.0004$) higher in breast tumors than in matched normal breast. A deleted *SRA* RNA was detected in most samples of normal breast and tumors. No differences occurred in the relative expression of the deleted *SRA* between normal breast and tumors. Within the breast tumor cohort the relative expression of the deleted *SRA* was positively correlated with tumor grade (Spearman $r = 0.556$, $p = 0.0135$) and size (Spearman $r = 0.655$, $p = 0.0023$). These data suggest that expression of core *SRA* is upregulated during breast tumorigenesis and that changes in the relative expression of a deleted *SRA* isoform occur during breast cancer progression. These data have been submitted for publication (16) see **appendix 3**).

Recently, a repressor of estrogen receptor activity (REA) also known as BAP37 was identified as a repressor of ER (10). It was relevant therefore to determine if REA was expressed in breast tissue and if so was its expression altered during tumorigenesis. We measured REA mRNA levels in normal and neoplastic human breast tissues. A single ~1.7 kb REA mRNA was identified by Northern blotting in breast cancer cells and biopsies. REA mRNA was measured by RT-PCR in ER+ breast tumors and matched adjacent normal tissue. Some breast tumors (12/19) contained higher levels of REA mRNA than their normal tissues,

but this was not statistical significant, although as shown previously *SRA* RNA, an ER coactivator, was significantly increased in tumors versus matched normal tissue in this set (16). REA mRNA levels varied amongst tumors and were found to be positively correlated (Spearman r=0.323, p=0.042) with ER level (determined by ligand binding) and inversely correlated with grade (Spearman r=-0.438, p = 0.0054). However, estrogen did not regulate REA mRNA steady state levels in MCF7 cells and progestins did not regulate REA mRNA steady state levels in T-47D human breast cancer cells in culture. These data suggest that while REA expression may not be altered in early breast tumorigenesis, altered regulation may contribute to breast cancer progression. These data are being written up in a manuscript and have been submitted to AACR in abstract form (17), see **appendix 4**).

These data suggest that alteration of factors, which can modulate ER signal transduction, occur during breast tumorigenesis and provide a very strong rationale to determine in parallel their expression during the early stages of pre-neoplastic progression that precede the onset of invasive breast cancer and determine if differences exist between normal women and patients who develop breast cancer, in order to assess a potential role in increasing the risk of invasive breast cancer. See attached published article and submitted manuscript and abstracts, in **appendices 2, 3 and 4**.

7. KEY RESEARCH ACCOMPLISHMENTS.

* identification of initial patient cohort for study.

* retrieval, review and collection of appropriate tissues blocks for analyses.

* analysis of SRA expression in human breast tissues showing that SRA expression is increased during human breast tumorigenesis.

* analysis of SRA expression in human breast tumors showing that SRA-like expression varies amongst human breast tumors and may be altered during breast cancer progression.

*analysis of REA expression in human breast tissues showing that little alteration of REA expression occurs during breast tumorigenesis but its expression in breast tumors correlates with markers of good prognosis i.e. estrogen receptor expression and low grade.

8. REPORTABLE OUTCOMES.

1. Leygue E, Dotzlaw H, Watson PH, Murphy LC (1999) Expression of the steroid receptor RNA activator (SRA) in human breast tumors. Cancer Res 59: 4190-4193. See **appendix 2**

2. Leygue E, Dotzlaw H, Simon SR, Watson PH, Murphy LC (submitted) Increased expression of the steroid receptor RNA activator (SRA) during human breast tumorigenesis. See **appendix 3**

3. Simons SLR, Parkes A, Leygue E, Dotzlaw H, Watson PH, Murphy LC (submitted) Expression of a repressor of estrogen receptor (ER) activity (REA) in human breast tissues. 91st Annual Meeting of the AACR, San Francisco, CA, April 1-5, 2000. See **appendix 4**

9. CONCLUSIONS.

Our previous results together with our current results suggest that estrogen receptor mediated signal transduction is complex and multifaceted in human breast cancer. Our previous data suggested that altered expression of estrogen receptor-α, estrogen receptor-β and their variants occurs during breast tumorigenesis. Our current data suggest that expression of cofactors which enhance estrogen receptor activity, e.g. SRA, is increased during breast tumorigenesis while expression of cofactors which can repress estrogen receptor activity, e.g. REA, either does not change or is not increased to a similar extent during breast tumorigenesis. Overall the data support the hypothesis that a substantial alteration of estrogen signaling pathways occurs during human breast tumorigenesis. In addition both our previous and our current data provide a very strong rationale to determine in parallel estrogen receptor isoform/variant expression and cofactor expression during the early stages of pre-neoplastic progression that precede the onset of invasive breast cancer and determine if differences exist between normal women and patients who develop breast cancer, in order to assess a potential role in increasing the risk of invasive breast cancer.

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14. H. Endoh, et al., *Mol Cell Biol* **19**, 5363-5372. (1999).
15. E. Leygue, H. Dotzlaw, P. Watson, L. Murphy, *Cancer Res* **59**, 4190-4193 (1999). **Appendix 2.**
16. E. Leygue, H. Dotzlaw, S. Simon, P. Watson, L. Murphy, (submitted). **Appendix 3.**
17. S. Simons, et al., *AACR* **91st Annual Meeting**, San Francisco, CA (2000 (submitted)). **Appendix 4.**

APPENDIX 1

patient#	lab#	AgePM (>=55)	Age	INVASIVE	INV+IS	IS	ADH	Typical DH	FCC	N
210	97s1459-9	1	62	A	F					H3
457	97s1858-6	1	91	A						I
659	97s2822-2	1	67	A	A				C,E	
749	97s7771-4	1	75	A	A					G
967	97s4721-8	1	81	A						
1821	97s8758-4	1	81	A						
1998	97s8743-6	1	61	A	A					
2686	98-s769-8	1	66	A	M	G,C,F,K,J				Q,I
2439	98-s2875-8	1	62	A,A					H1,G	
2439	97-s10898-4	1	62	A,B1					B4,E	
1502	97s7008-5	1	57	A,F	F,A				C	
1919	97s8756-0	1	64	A,G2	G2					
642	97s127-6	1	76	A1	A5			A4,A5		
1436	97s6303-6	1	78	A1	A1		A1	A1,B1	B1	
1843	97s8692-3	1	57	A1	A1				B2	
2045	97s9417-9	1	65	A1				C3		
2621	97-s12739-6	1	79	A1					A1	A6
2624	97-s12732-7	1	73	A1	A5		A5	A1,A6-2	B3	
451	97s125-2	1	88	A1,A3	A5			A3,A5	B	
1443	97s6282-2	1	68	A1-1	A1	B2, A3-2	B2,A3-2		A6	A3
2055	97s9298-8	1	78	A2			A2,B			D
393	97s1393-8	1	63	A3	A3					A5
3210	98-3982	1	80	A3						B1
2468	97-s11703-8	1	81	A3,A2		G,D	D	G	C	
1096	97s5059-3	1	67	A3,A5			D5	A3,D2,D4		
724	97s1400-1	1	55	A4	A4					A12
1538	97s7560-2	1	70	A4		A5-1				
510	97s1601-0	1	67	A5		B,F		A5		
1442	97s6325-6	1	56	A5	A5				A4	A4
3562	98-5401	1	67	A5	A1					A4
2861	98-s1678-0	1	65	AA		AA				
2909	98-5417	1	63	AA		B11				B11
1028	97s4591-7	1	61	B	B			B		B
1546	97s7476-6	1	75	B	B					
1716	97s8209-1	1	69	B						
2438	97-s11006-2	1	71	B	B					
1898	97s8657-6	1	67	B2				D		
896	97s3756-5	1	57	B3,C3	B3,C3	DX2				D
2613	97-s11715-5	1	88	B4						D4
1555	97s7679-9	1	72	B7		C3		B2		
1822	97s8690-9	1	55	C						
1556	97s7633-5	1	72	C3	C3					
392	97s1594-7	1	80	C4						B
3145	98-s2714-2	1	65	D	C1,C2	E3,E4		E1,C1,C2	E5	
1435	97s6619-4	1	73	D,E	D		E			
496	97s1855-5	1	58	DCIS		A,D				B
203	97s2848-0	1	66	E	G					M
2462	97-s12384-2	1	63	E				H1,G6	H2,G7	I3
2656	97-s11708-3	1	56	E1,E3	F4				F1	B
2618	97-s12815-3	1	59	E1,E4,E5	E1,E4,E5					E6

NODE MET	invasive	surgery-date	peration-type1
	D-INF	7-Feb-97	8543
	D-INF	18-Feb-97	8521
	D-INF	17-Mar-97	8521
	D-INF	29-Jul-97	8512
	D-INF	8-May-97	8543
	D-INF	2-Sep-97	8512
	T-INF	2-Sep-97	8521
	D-INF	23-Jan-98	8543
	L-INF	24-Mar-98	8521
	D-INF+T-INF	28-Oct-97	8512
I3	D-INF	7-Jul-97	8512
	D-INF	2-Sep-97	8521
	D-INF+COL-INF	6-Jan-97	8512
	D-INF	17-Jun-97	8521
	D-INF	29-Aug-97	8521
D2	D-INF	19-Sep-97	8521
	T-INF	15-Dec-97	8512
	D-INF	15-Dec-97	8521
	D-INF	6-Jan-97	8541
	D-INF	17-Jun-97	8521
	COL-INF	16-Sep-97	8521
	D-INF	6-Feb-97	8521
	D-INF	28-Apr-98	8521
I4	D-INF	18-Nov-97	8543
D7-1,D7-3	D-INF	20-May-97	8512
	D-INF	6-Feb-97	8543
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	D-INF	30-Oct-97	8521
	D-INF	29-Aug-97	8521
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	D-INF	29-Aug-97	8512
	D-INF	24-Jul-97	8521
	D-INF	12-Feb-97	8543
H3	D-INF	19-Mar-98	8512
	D-INF	24-Jun-97	8521
	D-INF	18-Feb-97	8512
	IV-OT	17-Mar-97	8543
	IV-OT	5-Dec-97	8521
	D-INF	19-Nov-97	8523
G4,G3,G5	D-INF	16-Dec-97	8521

1434	97s6611-8	1	69	E1-3,D	E1-3,D			A		B1
209	97s1443-4	1	74	E14				C	D	
2437	97-s10625-0	1	69	E3,A5	E2,D4					F
299	97s1234-6	1	59	E7	E7				E7	
2627	97-s11411-4	1	56	F1,G1	F1,G1		G3,I1	K,H,D2,C3,E3	D2,E2	
1437	97s6639-0	1	72	F5	A5			A1		
2600	97-s11870-9	1	71	G						
1463	97s6381-6	1	58	H	H			K	K	J,I
2634	97-s11381-4	1	72	K,L,M					FS1	I,D
602	97s2342-3	1	80	L		G		C2,C5	C2,C5	H4
2631	97-s11398-6	1	84	M,J	C,E				N	
203	97s1728-6	1	66	PN						
2637	98-s427-9	1	57	PN						
3110	98-s1795-6	1	72	PN						
3155	98-s2757-5	1	83	PN						
2986	98-s1675-9	1	87	PNX2						
2098	97s9728-2	1	82	T3		B		F1		
298	97s1250-4	1	55		D	E4			B	A
389	97s1434-8	1	75		A					B
512	97s898-1	1	74							
694	96s13076-7	1	66							
724	97s1121-1	1	55							
896	97s4623-1	1	57						A,B	
967	97s4243-3	1	81							
1053	97s4892-7	1	58							
1053	97s7980-2	1	58							
1143	97s5186-2	1	65							
1186	97s5735-6	1	87							
1454	97s6510-0	1	64							
1953	97-s10478-4	1	68		B2			C2		B4
2107	97s9566-8	1	87							
2453	97-s10695-1	1	63							
2602	97-s12170-9	1	77							
2602	97-s12469-2	1	77							
2624	97-s11336-4	1	73							
2635	97-s11112-8	1	60		E1,E2					G2
2685	98-s361-8	1	72		C	C				
2695	98-s963-8	1	62							
2858	98-s1701-1	1	95		A1	A2,A3,B				B
3027	98-s653-2	1	68		11	10(LCIS)	7	8		5
3028	98-s523-2	1	73		A,K	J,L,M		G,P7		
3146	98-s2849-7	1	77		B2					D3
3212	98-s3033-1	1	74		A,B,D,E					
3271	98-s3319-0	1	65		1					
3538	98-5152	1	77		B4,A2-1	B2,A1	B1	A2-3	A4-3	
3655	98-5192	1	73							
3655	98-6408	1	73							
3676	98-5447	1	73							
3754	98-6194	1	66							
3760	98-5145	1	60							
3760	98-6099	1	60							

	D-INF	24-Jun-97	8521
	D-INF	7-Feb-97	8543
	L-INF	21-Oct-97	8521
	L-INF	3-Feb-97	8521
	D-INF	10-Nov-97	8512
	D-INF	24-Jun-97	8512
	D-INF	24-Nov-97	8521
O2	D-INF	19-Jun-97	8512
	D-INF	10-Nov-97	8521
	COL-INF+D-INF	3-Mar-97	8512
	D-INF+L-INF	10-Nov-97	8521
	IV-OT	14-Feb-97	8512
	D-INF	14-Jan-98	8512
	D-INF	19-Feb-98	8512
	D-INF	20-Mar-98	344
	L-INF	17-Feb-98	8512
	COL-INF+IV-OT	26-Sep-97	8521
	D-INF	3-Feb-97	8521
	D-INF	7-Feb-97	8543
	D-INF	24-Jan-97	344
	IV-OT	6-Dec-96	8512
	D-INF	30-Jan-97	8512
	D-INF	6-May-97	8541
	D-INF	25-Apr-97	8512
	IV-OT	14-May-97	8512
	D-INF	5-Aug-97	8521
	D-INF	22-May-97	8512
	D-INF	4-Jun-97	8512
	D-INF	20-Jun-97	8521
F4	D-INF	16-Oct-97	8543
	L-INF	23-Sep-97	8512
	D-INF	22-Oct-97	8512
	D-INF	1-Dec-97	8512
	D-INF	8-Dec-97	8543
	D-INF	7-Nov-97	8512
I4	D-INF	3-Nov-97	8521
	D-INF	13-Jan-98	8543
	D-INF	28-Jan-98	8512
	D-INF+COL-INF	17-Feb-98	8521
	L-INF	20-Jan-98	8512
Q	D-INF	16-Jan-98	8521
EX2	D-INF	24-Mar-98	8543
	D-INF+T-INF	30-Mar-98	8512
	L-INF	8-Apr-98	8512
	COL-INF	1-Jun-98	8521
	L-INF	2-Jun-98	8512
	L-INF	10-Jul-98	8521
	COL-INF	10-Jun-98	8512
	D-INF	30-Jun-98	8521
	T-INF	1-Jun-98	8512
	T-INF	26-Jun-98	8521

3766	98-6245	1	81							
3830	98-5181	1	63							
3888	98-6153	1	61							
3896	98-5805	1	65							
3364	98-s3489-2		1	1						5
2553	97-s11579-9		54	1,2	1,2	5				3
515	97s1790-1		48	A				F2	F2,F1	F4
3073	98-s2323-4		45	A						
3111	98-s1308-6		36	A						
684	97s2396-6		47	A,B						B2
969	97s4559-1		52	A1					A8	A1,A8
2996	98-s655-6		40	A1						C1
396	97s1551-4		50	A1,A3	A1,A3				A6,A7,B	B
2095	97s9149-9		52	A2		A4,B2				A18
1468	97s6655-8		47	A4,A1	A1			D2	D1,A2	
1501	97s6986-7		53	A5	A5				A6	
3162	98-s2074-3		49	B,A						
1752	97s8709-6		39	B3	B3,C2					C4
2097	97s9385-5		52	B4					E1	
2180	97-s10467-4		40	B4,B5,B3	B4,B5,B3	B2,B,E				D5,D2
2064	97s9510-1		47	C	C	E				G
519	97s2608-6		45	C2		D3				D1
1966	97s8895-6		51	C7	C7					
3144	98-s1179-2		53	D				B3,A,C	B1,B5	A,C
1934	97s9017-5		52	D3	D3		D3	J1,J4		
2174	97-s9776-3		54	F2,F1,A				A	C	
2422	97-s11323-0		45	FS2,A1-6	FS2					A1-3
739	97s2844-2		39	G	G		G(other)			D
2992	98-s1649-8		37	G2						D
2179	97-s11186-7		53	L,K						I
1176	97s5376-1		52	PHYLOIDES						
483	97s1853-1		36							
483	98-s2287-9		36							
647	97s6253-0		46							
1115	97s12163-7		36							
2032	97-s11222-2		42							
2056	97s9126-2		52							
2467	97-y6988-1		51							
2707	97-s12267-6		41							
2993	98-s1529-1		50		B			K5	K3,C,L	
3111	98-s820		36							
3675	98-5325		54							
3677	98-5057		46							
3678	98-4530		36							
3702	98-4161		48							
3759	98-6107		46							
3902	98-5710		40							
	99-432			B1,B2,B3			E3	B4	E2	A1-2

	L-INF	3-Jul-98	8512
	D-INF	2-Jun-98	8512
	D-INF	29-Jun-98	8521
	D-INF	18-Jun-98	8512
	D-INF	14-Apr-98	8512
	D-INF	14-Nov-97	8512
	D-INF	17-Feb-97	8512
	D-INF+L-INF	10-Mar-98	8512
	D-INF+L-INF	5-Feb-98	8512
	D-INF	4-Mar-97	8512
	T-INF	5-May-97	8521
	D-INF	20-Jan-98	8512
	D-INF	11-Feb-97	8521
	D-INF	12-Sep-97	8521
F3	D-INF	24-Jun-97	8543
	D-INF	7-Jul-97	8521
	D-INF	2-Mar-98	8512
I2	D-INF	2-Sep-97	8512
F2	D-INF+L-INF	18-Sep-97	8543
	D-INF	16-Oct-97	8543
	COL-INF	22-Sep-97	8512
J3	D-INF	11-Mar-97	8512
	D-INF	5-Sep-97	8521
	D-INF		8512
	D-INF	9-Sep-97	8521
K,L,N	D-INF	29-Sep-97	8543
	D-INF+L-INF	7-Nov-97	8512
W	D-INF	17-Mar-97	8512
J1	D-INF	16-Feb-98	8521
	D-INF	4-Nov-97	8543
	IV-OT	27-May-97	8521
	D-INF	18-Feb-97	8521
	D-INF	9-Mar-98	8541
	D-INF	16-Jun-97	8512
	D-INF/L-INF	15-Nov-96	8512
	D-INF	5-Nov-97	8512
	D-INF	11-Sep-97	8521
	D-INF	22-Oct-97	8511
	IV-OT	3-Dec-97	8512
	D-INF	12-Feb-98	8512
	D-INF+L-INF	19-Feb-98	8543
	D-INF	5-Jun-98	8512
	D-INF	28-May-98	8521
	D-INF	13-May-98	8543
	D-INF	4-May-98	8541
	D-INF	26-Jun-98	8521
	D-INF	16-Jun-98	8521

Tub

APPENDIX 2

[CANCER RESEARCH 59, 4190–4193, September 1, 1999]

Advances in Brief

Expression of the Steroid Receptor RNA Activator in Human Breast Tumors[1]

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Abstract

The expression of the recently described steroid receptor RNA activator (*SRA*) was measured by semiquantitative reverse transcription-PCR within 27 independent breast tumors, spanning a wide spectrum of grade and estrogen receptor (ER) and progesterone receptor (PR) levels. Subgroup analysis showed that *SRA* expression was similar in ER+/PR+ (median = 65.5, $n = 8$) and in ER−/PR− (median = 94.6, $n = 5$) tumors. Interestingly, *SRA* expression in these two subgroups was significantly (Mann-Whitney rank-sum test, $P < 0.05$) lower than that observed in ER+/PR− (median = 156.4, $n = 6$) and ER−/PR+ (median = 144.8, $n = 8$) tumors. A variant form of *SRA*, presenting a deletion of 203 bp within the *SRA* core sequence, was also observed in breast tumor tissues. The relative expression of this new *SRA* isoform correlated with tumor grade (Spearman coefficient $r = 0.53$, $n = 27$, $P = 0.004$). These data suggest that changes in the expression of *SRA*-related molecules occur during breast tumor progression.

Introduction

Estrogens, through their mitogenic action on breast epithelial cells, regulate the growth and the development of normal as well as neoplastic human mammary tissue (1). The ability of antiestrogens such tamoxifen or raloxifene to antagonize this estrogenic action provides the basic rationale for endocrine therapy and prevention (for review see Ref. 2). Estrogen action is mainly mediated through two ERs,[3] ER-α and ER-β (3–5), which belong to the steroid/thyroid/retinoic acid receptors superfamily (6) and act as ligand-dependent transcription factors. The mechanisms by which steroid receptors modulate the transcription of target genes is under extensive investigation (7). Once bound to the ligand, the receptors undergo conformational changes and dimers of receptors recognize specific regulatory DNA sequences upstream of target genes. Activated receptors, through interactions with coactivator proteins, direct the assembly and the stabilization of a preinitiation complex that will ultimately conduct the transcription of these genes (see Ref. 8 and references therein). To an already long list of nuclear receptor coactivators (8), which includes the p160 proteins (such as SRC-1 and AIB1), Lanz *et al.* (9) recently added the *SRA*. *SRA* differs from other coactivators in two main ways. (*a*) *SRA* transcripts do not appear to be translated, and therefore, this coactivator acts as an RNA and not as a protein. Lanz *et al.* (9) showed that *SRA* exists in a ribonucleoprotein complex that contains SRC-1 and is recruited by steroid receptors. (*b*) *SRA* appears to be actually specific for steroid receptors. Indeed, most of the receptor-interacting factors, such as SRC-1 or TIF2/hSRC-2, interact with and coactivate both class I and class II nuclear receptors (9). Because of the importance of ER signaling pathways in the mechanisms underlying breast tumor progression, it was important to establish whether *SRA* could be expressed in breast tumors. If so, it was also of interest to determine whether the expression of *SRA* was related to known markers of endocrine sensitivity and prognostic markers. We have selected a subset of breast cancer cases to look for possible correlations between *SRA* expression and already established predictive and/or prognostic factors, such as grade, ER, and PR status (10).

Materials and Methods

Human Breast Tumors. Twenty-seven cases were selected from the National Cancer Institute of Canada-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The cases were selected according to their ER and PR status, as determined by ligand binding assay. Tumors were classified as ER−/PR+ ($n = 8$; ER range, 5–9 fmol/mg protein; PR range, 51–271 fmol/mg protein), ER+/PR− ($n = 6$; ER range, 59–151 fmol/mg protein; PR range, 5–10 fmol/mg protein), ER−/PR− ($n = 5$; ER range, 0–2 fmol/mg protein; PR range, 0–8 fmol/mg protein), and ER+/PR+ ($n = 8$; ER range, 50–127 fmol/mg protein; PR range, 101–285 fmol/mg protein). These tumors covered a wide spectrum of grade (grades 4–9), determined using the Nottingham grading system (11). Patients were 49–87 years old.

RNA Extraction and RT-PCR. Total RNA was extracted from frozen breast tissue sections using Trizol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions and quantified spectrophotometrically. One μg of total RNA was reverse-transcribed in a final volume of 25 μl, as described previously (12).

Primers and PCR Conditions. The primers used consisted of *SRA*coreU primer (sense, 5′-AGGAACGCGGCTGGAACGA-3′, positions 35–53; GenBank accession no. AF092038) and *SRA* core L primer (antisense, 5′-AGTCTGGGGAACCGAGGAT-3′, positions 696–678; GenBank accession no. AF092038). PCR amplifications were performed and PCR products analyzed as described previously (12), with minor modifications. Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 15 μl, in the presence of 1.5 μCi of [α-^{32}P]dCTP (3000 Ci/mmol), 4 ng/μl each primer, and 0.3 unit of Taq DNA polymerase (Life Technologies, Inc.). Each PCR consisted of 30 cycles (30 s at 60°C, 30 s at 72°C, and 30 s at 94°C). PCR products were then separated on 6% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gels were dried and exposed for 1 h to a Molecular Imager-FX Imaging screen (Bio-Rad, Hercules, CA). Amplification of the ubiquitously expressed *GAPDH* cDNA was performed in parallel and PCR products separated on agarose gels stained with ethidium bromide as described previously (12). Identity of PCR products was confirmed by subcloning and sequencing, as reported previously (13).

Quantification of *SRA* Expression. Exposed screens were scanned using a Molecular Imager-FX (Bio-Rad), and the intensity of the *SRA* corresponding signal was measured using Quantity One software (Bio-Rad). Three independent PCRs were performed. To control for variations between experiments, a value of 100% was arbitrarily assigned to the *SRA* signal of one particular tumor (tumor 14) measured in each set of PCR experiments, and all signals were expressed as a percentage of this signal. In parallel, *GAPDH* cDNA was amplified and following analysis of PCR products on prestained agarose gels, signals were quantified by scanning using NIH Image 161/ppc software. Three

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[2] To whom requests for reprints should be addressed, at Department of Biochemistry and Molecular Biology, University of Manitoba, Winnipeg, Manitoba R3E OW3, Canada. Phone: (204) 789-3812; Fax: (204) 789-3900; E-mail: eleygue@cc.umanitoba.ca.

[3] The abbreviations used are: ER, estrogen receptor; SRA, steroid receptor RNA activator; PR, progesterone receptor; RT-PCR, reverse transcriptase-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

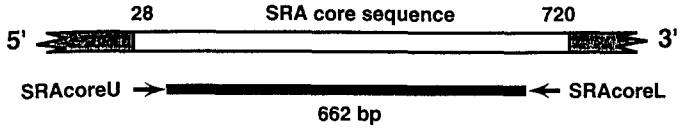

Fig. 1. *SRA* structure and primer presentation. *SRA* isoforms identified to date (9) differ their 5′ and 3′ terminal regions (■) but present an identical nucleotide sequence (□) in between (*SRA* core sequence, bases 28 to 720). *SRA*coreU and *SRA*coreL primers anneal with *SRA* core sequences and allow the amplification of a 662-bp fragment.

independent PCRs were performed. Each *GAPDH* signal was also expressed as a percentage of the signal observed in the tumor 14. For each sample, the average of *SRA* signal was then expressed as a percentage of the *GAPDH* signal (arbitrary units).

Quantification of *SRA*-Del Relative Expression. It has previously been shown that the coamplification of a wild-type and a deleted variant cDNA resulted in the amplification of two PCR products, the relative signal intensity of which provided a reliable measurement of the relative expression of the deleted variant (13, 14). For each sample, *SRA*-Del corresponding signal was measured using Quantity One software (Bio-Rad) and expressed as a percentage of the corresponding *SRA* signal. For each case, three independent assays were performed, and the mean was determined.

Statistical Analysis. Differences between tumor subgroups were tested using the two-sided Mann-Whitney rank sum test. Correlation between *SRA* expression and tumor characteristics was tested by calculation of the Spearman coefficient *r*.

Results

Detection of *SRA* and a Variant mRNA Deleted Form (*SRA*-Del) in Human Breast Tumor Tissues. The existence of three different *SRA* mRNAs have been reported (9). The sequences of these isoforms differ in their 5′- and 3′-terminal regions but are identical within their central region, called the core (Fig. 1). To investigate the expression of all described *SRA* isoforms in human breast tumor tissues, we designed primers to amplify a 662-bp fragment encompassing almost all of the *SRA* core region. Total RNA was extracted from 27 human breast tumors and reverse-transcribed, and PCR amplification was performed as described in the "Materials and Methods" using SRA core primers. A 662-bp fragment was obtained in all samples. However, the intensity levels varied from one sample to another (Fig. 2*A*). This fragment was sequenced and corresponded to the *SRA* core region. The differences in *SRA* expression were unlikely to result from different cDNA input, as shown by the similar intensities of *GAPDH* signal obtained after amplifying *GAPDH* mRNA in parallel using the same cDNAs (Fig. 2*B*). An additional fragment, migrating at an apparent size of 459 bp was also observed in most samples. Sequencing analysis revealed that this band corresponded to a variant form of *SRA* (referred to as *SRA*-Del) deleted in 203 bp between positions 155 and 357 (corresponding to GenBank accession no. AF092038).

The Expression of *SRA* Correlates with ER and PR Levels in Subgroups of Human Breast Tumors. For each case, the *SRA*-corresponding signal was quantified and expressed in arbitrary units, as described in "Materials and Methods." Results obtained from the 27 cases, grouped according to their ER and PR levels, as determined by ligand binding analysis, are presented Fig. 3*A*. When the cohort of cases was considered as a whole ($n = 27$), no correlation was observed between *SRA* expression and ER or PR levels. Indeed, similar levels of *SRA* were found in ER+/PR+ (median = 65.5, $n = 8$) and ER−/PR− (median = 94.6, $n = 5$) tumors (Fig. 3*A*). However, when only ER− tumors were considered ($n = 13$), a trend toward a positive correlation between *SRA* expression and PR levels was observed (Spearman coefficient $r = 0.527$, $P = 0.064$). *SRA* expression was higher in ER−/PR+ ($n = 8$, median = 144.8) than it was in ER−/PR− tumors (Fig. 3*A*); this difference was statistically significant (two-sided Mann-Whitney rank sum test, $P = 0.045$). In contrast, within ER+ cases ($n = 14$), *SRA* expression negatively correlated with PR levels (Spearman coefficient $r = -0.810$, $P = 0.0004$). *SRA* expression was higher (two-sided Mann-Whitney rank sum test, $P = 0.001$) in ER+/PR− ($n = 6$, median = 156.4) than it was in ER+/PR+ cases. In a similar way, *SRA* expression correlated positively (Spearman coefficient $r = 0.735$, $P = 0.009$) and negatively (Spearman coefficient $r = -0.532$, $P = 0.033$) with ER levels in PR− ($n = 11$) and PR+ ($n = 16$) cases, respectively. *SRA* levels were higher in ER+/PR− than in ER−/PR− tumors (two-sided Mann-Whitney rank sum test, $P = 0.017$) and in ER−/PR+ than in ER+/PR+ cases (two-sided Mann-Whitney rank sum test, $P = 0.047$). *SRA* levels of expression did not correlate with tumor grade scores (Fig. 3*B*).

The Expression of *SRA*-Del Correlates with Breast Tumor Grade Scores. For each case, *SRA*-Del signal was measured and expressed relative to the corresponding *SRA* signal, as described in the "Materials and Methods." *SRA*-Del relative signal did not correlate with ER or PR levels when the cohort of cases was considered as a whole or when ER−, ER+, and PR− subgroups were analyzed. Interestingly, *SRA*-Del expression positively correlated (Spearman coefficient $r = 0.512$, $P = 0.042$) with PR levels in PR+ subgroup ($n = 16$). However, no statistically significant differences (Fig. 4*A*) were observed between ER−/PR+ ($n = 8$, median = 2.346), ER+/PR− ($n = 6$, median = 2.561), ER−/PR− ($n = 5$, median = 6.571) and ER+/PR+ ($n = 8$, median = 3.528). By contrast, *SRA*-Del levels strongly correlated (Spearman coefficient $r = 0.530$, $P = 0.004$) with Nottingham grade scores within the whole cohort ($n = 27$). The level of expression of *SRA* was significantly higher (two-sided Mann-Whitney rank sum test, $P < 0.05$) in tumors of high grade ($n = 7$, median = 6.572) than it was in tumors of low ($n = 4$, median = 2.192) or intermediate ($n = 9$, median = 2.588) grade (Fig. 4*B*).

Discussion

Using primers annealing with the core region of the three previously described *SRA* isoforms (9), we have investigated *SRA* expression in 27 independent breast tumors by means of semiquantitative RT-PCR. These *SRA* isoforms, although different in their 5′- and 3′-terminal regions, are all able to coactivate steroid receptor. Indeed,

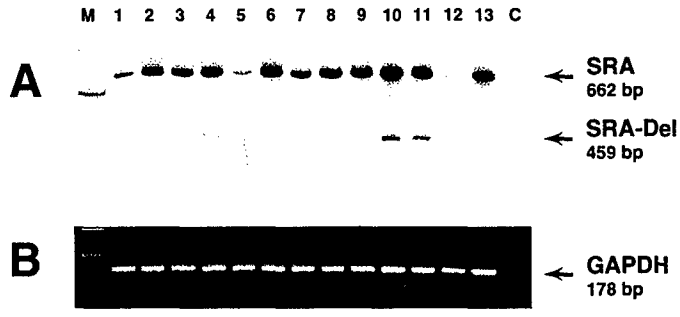

Fig. 2. Detection of *SRA* in human breast tumors by RT-PCR. Total RNA was extracted from 27 breast tumors, reverse-transcribed, and analyzed by PCR as described in "Materials and Methods." PCR products were separated on 6% acrylamide gels. Gels were dried and exposed 1 h to a Molecular Imager-FX Imaging screen. Screens were then scanned using a Molecular Imager-FX. *A*, computerized image showing the results obtained for 13 cases (*Lanes 1–13*). *Lane M*, molecular weight marker (ϕx174 RF DNA/*Hae*III fragments). *Lane C*, control lane, no cDNA added in the PCR. Sequencing analysis of PCR fragments revealed that the 662-bp (*SRA*) and 459-bp (SRA-Del) fragments corresponded to *SRA* and to a variant *SRA* isoform deleted in sequences from position 155 to 357 (GenBank accession no. AF092038), respectively. *B*, ethidium bromide-stained gel of the RT-PCR analysis of *GAPDH* mRNA run in parallel for the same samples.

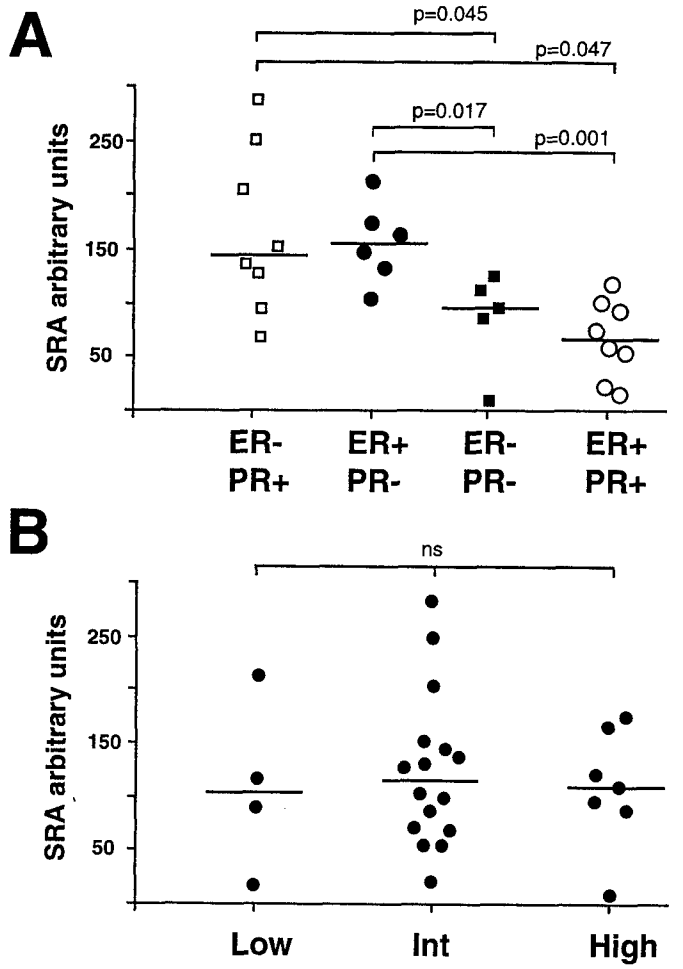

Fig. 3. Subgroup analysis of *SRA* expression within 27 human breast tumors. For each case, *SRA* expression was quantified and expressed in arbitrary units as described in "Materials and Methods." *A*, tumors were grouped according to their ER and PR status, as determined by ligand binding assay. □, ER−/PR+ tumors; ●, ER+/PR− tumors; ■, ER−/PR− tumors; and ○, ER+/PR+ tumors. *B*, tumors were grouped according to their grade: low (Nottingham grading scores 4–5), intermediate (Nottingham grading scores 6–7), and high (Nottingham grading scores 8–9). The *horizontal line* represents the median value in each group. *Ps* (two-sided Mann-Whitney rank sum test) are indicated when subgroups were statistically different. *ns*; no statistically significant differences were found between subgroups.

SRA core region was found to be necessary and sufficient for the coactivation properties of *SRA* isoforms (9). Therefore, although PCR performed using primers spanning the *SRA* core region is likely to recognize several different *SRA*-like molecules, the signal obtained corresponds to molecules that should all have essentially the same function, *i.e.*, coactivation of steroid receptors.

The expression of *SRA* did not correlate with ER or PR status when the cohort was considered as a whole. This differs from what has been observed for another coactivator, *AIB1*. Indeed, Anzick *et al.* (15) first showed that a strong expression of *AIB1* that resulted from *AIB1* gene amplification was observed in ER+ but not in ER− breast cancer cell lines. More recently, Bautista *et al.* (16) reported that *AIB1* gene amplification correlated with ER and PR positivity. Our results suggest that the pattern of expression of *SRA* is more complex. Indeed, we found that *SRA* expression could correlate positively or negatively with ER and PR levels, depending on the subgroup considered. The general trend appeared to be that, in tumors expressing a low level of one receptor (ER or PR), a positive correlation was found between *SRA* expression and the second receptor (PR or ER). Inversely, in tumors highly expressing one receptor (ER or PR), *SRA* expression negatively correlated with the level of expression of the second receptor (PR or ER). At this stage of the knowledge of *SRA* biological function, the interpretation of such an observation is difficult. Indeed, *SRA* has been shown to be able to coactivate both ER and PR (9). Moreover, progestins are known to decrease the steady state levels of ER-α mRNA and protein, whereas estrogens increase PR expression (17, 18). Therefore, all combinations and cross-talk appear possible. One could speculate that increased levels of *SRA* in ER−/PR+ cases could partially be responsible, by "boosting" the activity of the weakly expressed ER, of the expression of PR in these tumors. Inversely, in the same ER−/PR+ cases, the strong *SRA* expression could be responsible for an increased down-regulation of ER by PR. Our results suggest that *SRA* expression varies from one particular tumor to another. Changes in *SRA* expression can be associated with known prognostic and predictive factors such as ER and PR in particular tumor subgroups. The question of a direct involvement of *SRA* in the hormonal status changes occurring during breast tumor progression remains unanswered. Also of interest is the fact that *SRA* interacts with the activation function 1 of the steroid receptors (9). Activation function 1 is thought to mediate the agonistic effect of antiestrogens such hydroxytamoxifen (19). This agonistic action of antiestrogens is believed to be involved in part in the mechanisms underlying hormone

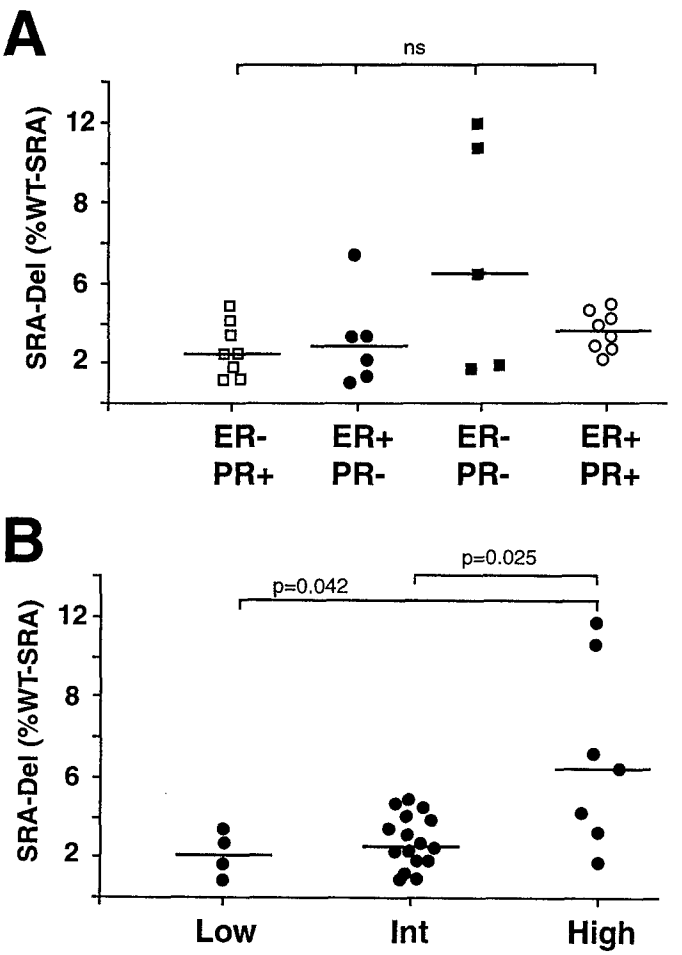

Fig. 4. Subgroup analysis of *SRA*-Del relative expression within 27 human breast tumors. For each case, SRA-D3 expression relative to *SRA* was quantified as described in "Materials and Methods." *A*, tumors were grouped according to their ER and PR status, as determined by ligand binding assay. □, ER−/PR+ tumors; ●, ER+/PR− tumors; ■, ER−/PR− tumors; and ○, ER+/PR+ tumors. *B*, tumors were grouped according to their grade: low (Nottingham grading scores 4–5), intermediate (Nottingham grading scores 6–7), and high (Nottingham grading scores 8–9). The *horizontal line* represents the median value in each group. *Ps* (two-sided Mann-Whitney rank sum test) are indicated when subgroups were statistically different. *ns*; no statistically significant differences were found between subgroups.

resistance in breast cancer. One could speculate that the level of *SRA* expression might, therefore, modulate and predict the response of a given tumor to hormone therapy. This hypothesis appears to be refuted by the observation of similar levels of *SRA* in ER+/PR+ and ER−/PR− tumors. But ER+/PR+ tumors, as opposed to ER−/PR− tumors, are likely to respond to endocrine therapy and prevention (see Ref. 2 and references therein). In these cases, the differences in ER levels rather than in *SRA* expression are more likely involved in the mechanisms underlying endocrine sensitivity. On the other hand, the observation of a higher *SRA* expression within ER−/PR+ cases, which are more likely to respond to hormone therapy than ER−/PR− tumors (see Ref. 20 and references therein), would be consistent with the hypothesis of a possible involvement of *SRA* in these mechanisms under some circumstances. One should also note that Berns *et al.* (21) recently reported that, although no correlation was found between the expression of *SRC-1* and ER status, a high expression of this coactivator indicated a favorable response to tamoxifen of patients with recurrent breast cancer. This issue can only be addressed in studies performed on tumors from patients that did and did not respond to endocrine therapy.

We have identified in breast tumor cases a new *SRA* isoform deleted in sequences from nucleotide 155 to 357 (*SRA*-Del). Interestingly, sequence comparison using the BLAST algorithm and the human EST database showed that this deleted *SRA* isoform has already been found in a pooled cDNA library containing cDNAs from melanocyte, fetal heart, and pregnant uterus (GenBank accession no. AA426601). Because uterus is another steroid target tissue, it could be hypothesized that the source of *SRA*-Del in this pooled library was, indeed, uterus. Even though the structure of the *SRA* gene has not yet been published, *SRA*-Del appears to correspond to a perfect exon-3 deleted *SRA* variant. *SRA* gene has recently been located on chromosome 5q31.3-32.[4] Sequence analysis of the corresponding DNA sequence (chromosome 5, BAC clone 319C17; GenBank accession no. AC005214) revealed that the fragment from nucleotide 155 to 357 corresponds to the third *SRA* exon. The putative function of *SRA*-Del remains to be determined. One should, however, note that a recombinantly developed *SRA* mutant, deleted of the region 3′ of a *Bbs*I site (position 341) and, therefore, partially deleted of exon 3 sequences, did not coactivate steroid receptors (9). Moreover, exon 3 deletion introduces a shift in the open reading frames, suggested by Lanz *et al.* (9), and could lead to a premature termination of the putative SRA proteins. One could, therefore, hypothesize that *SRA*-Del might interfere with *SRA* activity. The resulting modifications of the steroid receptor signaling pathways could confer a more aggressive behavior to the tumors expressing higher levels of *SRA*-Del. The positive correlation between *SRA*-Del levels and tumor grade scores would be consistent with this hypothesis.

Interestingly, modifications of the long arm of the chromosome 5 have been reported in breast tumors. Indeed, Hermsen *et al.* (22) found a frequent chromosomal gain in 5q within a subset of 53 lymph node-negative breast carcinoma, whereas Schwendel *et al.* (23) observed a frequent loss of this region in 39 invasive breast carcinomas. Moreover, among *BRCA1* mutation carriers, loss of 5q was observed more frequently than in the control patient (24). One could, therefore, speculate that the loss of *SRA* is selected for during tumor progression in cells lacking *BRCA1* functional gene. Whether changes in *SRA* expression result from chromosomal abnormalities remains to be determined.

In conclusion, we have shown that *SRA* is expressed in breast tumors and that its expression correlates with ER and PR levels in particular tumor subgroups. We speculate that changes in *SRA* expression could be involved in the mechanisms underlying tumor progression and hormone resistance.

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[4] http://www.ncbi.nlm.nih.gov/genemap/loc.cgi?ID=12637.

APPENDIX 3

Increased Expression of the Steroid Receptor RNA Activator (SRA) During Human Breast Tumorigenesis.

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Keywords: SRA, steroid hormone receptor activator, normal breast, breast tumorigenesis.

Abstract.

Background: SRA, a novel steroid receptor coactivator, which is active as an RNA molecule has recently been identified. Furthermore, we have previously detected the expression of SRA RNA in human breast tumor biopsy samples, which suggested that it could be involved in regulation of steroid hormone receptor activity in human breast cancers. ***Purpose:*** Since estrogen receptor-α expression is upregulated in human breast tumorigenesis it was of interest to determine if SRA was expressed in normal human breast tissue and if so was its expression altered in human breast tumors. ***Methods:*** Using reverse transcription polymerase chain reaction assays, expression of SRA was compared between adjacent normal human breast tissue and matched breast tumors from 19 patients. ***Results:*** Core SRA RNA was detected in both normal and neoplastic breast tissues. The level of SRA RNA was found to be significantly (Wilcoxon matched pairs test, $p = 0.0004$) higher in breast tumors than in the matched normal breast. A deleted SRA RNA was detected in most samples of normal and neoplastic breast tissues. No differences occurred in the relative expression of the deleted SRA between normal breast and tumors. However, within the breast tumor cohort the relative expression of the deleted SRA was positively correlated with tumor grade (Spearman coefficient, $r = 0.556$, $p = 0.0135$) and size ($r = 0.655$, $p = 0.0023$). ***Conclusion:*** Core SRA RNA and a deleted SRA RNA are present in both normal and neoplastic human breast tissues. The expression of SRA is significantly increased in breast tumors compared to the adjacent normal breast tissue. The relative expression of the

deleted SRA was increased in those tumors with markers of a poorer prognosis. ***Implications:*** These data suggest that expression of core SRA is upregulated during breast tumorigenesis and could contribute to altered steroid receptor activity in breast tumors, which may in turn effect hormone responsiveness. Further, changes in the relative expression of a deleted SRA isoform occur during breast cancer progression and may have some role in breast cancer progression.

Introduction.

Recently, a novel steroid receptor coactivator, SRA (*1*) was isolated and characterized. Interestingly, the available data suggest that the SRA RNA transcript is the functionally important molecule with respect to its steroid receptor activator activity (*1*) and is found within ribonucleoprotein complexes which may also contain other steroid receptor activators such as SRC-1 (*2*) which are functional as proteins. Previously, we have detected the expression of SRA RNA in human breast tumor biopsy samples (*3*). In contrast to another steroid receptor activator, AIB1, whose overexpression is correlated with estrogen (ERα) and progesterone receptor (PR) expression (*4*) SRA expression was not correlated overall with steroid receptor status as measured by ligand binding assays (*3*). Although the expression of a deleted form of SRA RNA was found to correlate with increasing tumor grade, the total expression of SRA-like RNA was unrelated to markers of progression and endocrine sensitivity in human breast tumors overall (*3*). These previous results, however, did not address the possibility that SRA expression could be altered during breast tumorigenesis. Since estrogen receptor signaling is thought to be altered during breast tumorigenesis as well as breast cancer progression, we have tested the hypothesis that altered expression of SRA is associated with breast tumorigenesis and therefore could contribute to altered estrogen signaling during breast tumorigenesis.

Materials and Methods.

Human Breast Tissues.

Nineteen ER positive primary human breast tumor biopsies (ER positivity defined as > 3 fmol/mg protein in classical ligand binding assays) were selected from the National Cancer Institute of Canada-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The ER levels ranged from 3.7 - 83 fmol/mg protein and the PR levels ranged from 2.7 - 112 fmol/mg protein (PR positivity defined as > 10 fmol/mg protein in classical ligand binding assays; 14 tumors were PR+ and 5 tumors were PR -). For each case, matched adjacent normal and tumor frozen tissue blocks were available. The quality of each block and the relative cellular composition was determined by the histopathological assessment of sections from adjacent mirror image paraffin-embedded tissue blocks, as previously described (*5*). The presence of normal ducts and lobules as well as the absence of any atypical lesion was confirmed in all normal tissue specimens. The tumors spanned a wide range of grades (grade scores 5-9) as determined by the Nottingham grading system (*6*), and ranged in size from 0.6 - 6.4 cm.

RNA Extraction and RT-PCR conditions.

Total RNA was extracted from 20 μm frozen tissue sections (20 sections per tumor; 35 sections for normal tissues) using Trizol™ reagent (Life Technologies, NY) according to the manufacturer's instructions and quantified spectrophotometrically. One μg of total RNA was reverse transcribed in a final volume of 25 μl as previously described (*7*).

Primers and PCR conditions.

The primers used were SRAcoreU primer (5'-AGGAACGCGGCTGGAACGA -3'; sense; positions 35-53, Genbank accession number AF092038) and SRAcoreL primer (5'- AGTCTGGGGAACCGAGGAT -3'; antisense; position 696-678, Genbank accession number AF092038). PCR amplifications were performed and PCR products analyzed as previously described (*7*) with minor modifications. Briefly, 1 µl of reverse transcription mixture was amplified in a final volume of 15 µl, in the presence of 1.5 µCi of (α-^{32}P) dCTP (3000 Ci/mmol), 4 ng/µl of each primer and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY). Each PCR consisted of 30 cycles (30 sec at 60°C, 30 sec at 72°C and 30 sec at 94°C). PCR products were then separated on 6% polyacrylamide gels containing 7M urea. Following electrophoresis, the gels were dried and exposed 2 hours to a Molecular Imager™-FX Imaging screen (Bio-Rad, Hercules, CA). Amplification of the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA was performed in parallel and PCR products separated on agarose gels stained with ethidium bromide as previously described (*7*). Identity of PCR products was confirmed by subcloning and sequencing, as previously reported (*8*).

Quantification of SRA expression.

Exposed screens were scanned using a Molecular Imager™-FX (Bio-Rad, Hercules, CA) and the intensity of the signal corresponding to SRA was measured using Quantity One™ software (Bio-Rad, Hercules, CA). Three independent PCRs were performed. In order to control for variations between experiments, a value of 100% was arbitrarily assigned to the SRA signal of one particular tumor (tumor #14) measured in each set of PCR experiments

and all signals were expressed as a percentage of this signal. In parallel, *GAPDH* cDNA was amplified and following analysis of PCR products on prestained agarose gels, signals were quantified by scanning using MultiAnalyst™ (Bio-Rad, Hercules, CA). Three independent PCRs were performed. Each *GAPDH* signal was also expressed as a percentage of the signal observed in the tumor #14. For each sample, the average of SRA signal was then expressed as a percentage of the *GAPDH* signal (arbitrary units).

Quantification of the relative expression of the deleted SRA variant RNA.

It has previously been shown that the co-amplification of a wild-type and a deleted variant cDNA resulted in the amplification of two PCR products, the relative signal intensity of which provided a reliable measurement of the relative expression of the deleted variant (*8,9*). For each sample, SRAdel corresponding signal was measured using Quantity One™ software (Bio-Rad, Hercules, CA) and expressed as a percentage of the corresponding core SRA signal. For each case, 3 independent assays were performed and the mean determined.

Statistical analysis

Differences between normal samples and their matched tumors were tested using the Wilcoxon matched pairs test, two-tailed. Correlation between SRA expression and tumor characteristics was tested by calculation of the Spearman coefficient r.

Results.

Detection of SRA and a deletion variant RNA form in both normal and neoplastic human breast tissues.

Using the SRA specific primers described in the "Materials and Methods" section, which amplify the core SRA sequences (Figure 1) as described by Lanz *et al.,* (*1*), we have previously detected two PCR products of 662 bp and 459 bp in human breast tumors (*3*). Cloning and sequencing revealed the identity of the 662 bp fragment with the SRA core region (*1*), and the 459 bp fragment as a variant form of SRA deleted in 203 bp between positions 155 and 357 (Figure 1, as numbered according to Genbank accession number AF092038). The current analysis identified the 662 bp product in all breast tissue samples assayed, both normal and neoplastic. As well, a 459 bp product corresponding to a SRA transcript deleted in 203 nucleotides was detected in the majority of tumors (n = 18) and normal samples (n = 17), and always together with the 662 bp product (Figure 2). Therefore, SRA core sequences are expressed in all human breast tissues and the expression of the deleted SRA transcript is not tumor specific.

Comparison of the expression of SRA and deleted SRA in adjacent normal breast tissue and matched primary breast tumors.

To determine whether alterations in core SRA expression occur during breast tumorigenesis, the expression of SRA RNA was measured in primary tumor tissues and their adjacent matched normal breast tissues from 19 different patients, as described in the Materials and Methods section. The analysis was confined to tissues from women whose breast tumor was ER+, as determined by ligand binding assays. Examples of the results obtained are shown in Figure 2. The expression of SRA corrected for the *GAPDH* signal in

each tissue sample for all the matched normal and tumor pairs is shown as a scatter graph in Figure 3A. The level of expression of core SRA was found to be significantly higher (Wilcoxon matched pairs test, $p = 0.0004$) in the tumor samples compared to their adjacent normal breast tissue. When the deleted SRA is detected, the expression of the variant SRA transcript relative to the core SRA expression was not significantly different between normal breast tissues and their matched adjacent breast tumors (Figure 3B). These data suggest that core SRA expression is upregulated during breast tumorigenesis, but the relative expression of a deleted SRA variant is not altered during breast tumorigenesis.

Correlation of SRA expression and the relative expression of deleted SRA with tumor characteristics.

The level of core SRA expression in the tumor cohort used in this study was not correlated with PR status, grade, tumor size or nodal status. However, the relative expression of the deleted SRA transcript in the tumors was positively correlated with grade score (Spearman $r = 0.556$, $p = 0.0135$), and tumor size (Spearman $r = 0.655$, $p = 0.0023$) but was not correlated with PR status or nodal status. These data suggest that increased relative expression of a deleted SRA variant is more likely to occur in those breast tumors with characteristics of a poorer prognosis, and may be associated with breast tumor progression.

Discussion.

SRA is a novel steroid hormone receptor activator. In particular it is different from other steroid receptor coactivators in three important ways: firstly it functions as a RNA molecule rather than a protein; secondly, it is specific for steroid hormone receptors; and thirdly, rather than effecting AF2 of steroid hormone receptors, it is specific for the AF1 domain of these receptors (*1*).

Since steroid hormones, in particular estrogen, play an important role(s) in the growth and function of both the normal and neoplastic human breast (*10,11*) and since altered estrogen receptor signaling likely occurs both in breast tumorigenesis and breast cancer progression (*12-15*), alteration of factors which may influence ER activity during breast tumorigenesis and/or progression are highly relevant to investigate in human breast tumorigenesis *in vivo* (*16,17*). Moreover, there are data which support increased expression of at least one steroid receptor activator, AIB1, in human breast cancers *in vivo* (*18*). However, AIB1 functions as a protein, and its site of action is associated with the ligand activated AF2 function of nuclear receptors generally (*18,19*). In contrast, SRA's site of action is the ligand independent AF1 function (*1*) of steroid hormone receptors, which in the case of ERα is thought to be important for cross-talk between growth factor/MAP kinase pathways (*20,21*), and is likely involved in mediating the agonist activity of antiestrogens such as tamoxifen. This underscores the importance of investigating factors which modulate specifically the AF1 activity of steroid hormone receptors in human breast tumorigenesis since marked alterations in estrogen and antiestrogen responsiveness are known to occur during breast tumorigenesis and breast cancer progression. Furthermore, since antiestrogens such as tamoxifen have recently been shown to be efficacious in the prevention of breast cancer (*22*), an understanding of the expression and role of factors likely to effect tissue responsiveness to estrogens and antiestrogens in human breast is absolutely necessary.

The data presented in our study suggest that the expression of the SRA is significantly increased in ER positive human breast tumors *in vivo* compared to their adjacent matched normal breast tissue. Since SRA is functional as a steroid receptor activator as a RNA molecule (*1*), measurement of the level of

SRA RNA likely reflects functional equivalents of this molecule in the breast tissues, and therefore our data support the hypothesis that the activity of this receptor activator is significantly upregulated during human breast tumorigenesis and therefore may have a role in increasing estrogen receptor signal transduction and sensitivity during breast tumorigenesis. Interestingly, our previous data (*3*) suggest that the level of expression of SRA in primary tumors, in contrast to AIB1 (*4*), is not correlated with ER and PR status overall, which suggests that SRA may subserve other functions in ER and PR negative tumors compared to ER and PR positive tumors. However, subgroup analysis within a previous cohort (*3*) showed that SRA expression correlated with either ER or PR depending on the subgroup considered. The general trend was that in tumors with a low level of expression of one receptor either ER or PR, a positive correlation was found between SRA and the second receptor, either PR or ER. In contrast in tumors expressing a high level of one receptor, either ER or PR, a negative correlation of SRA expression with the level of expression of the second receptor, either PR or ER was found. The functional significance of such correlations is unclear at this stage, but may reflect compensatory regulation under conditions of potentially reduced steroid responsiveness.

We have found no correlations of SRA expression with different percentages of epithelial and stromal cell types in either the normal or the tumor compartments of this cohort (data not shown), suggesting that differences in proportions of cell types and cellularity amongst the tissues is unlikely to explain the results obtained. Previously, SRA was shown to be expressed in human breast cancer cell lines in culture (*1*), consistent with the idea that SRA is expressed in epithelial cells. Our data are consistent with this observation and together the data support the hypothesis that the expression

of SRA is increased significantly in human breast cancer cells both *in vivo* and *in culture.*

At least three isoforms of SRA RNA, containing the so-called core region (see Figure1) but differing in the regions outside of the core region, both 5' and 3', were reported previously (*1*). It is the core region of SRA which is necessary and sufficient for the steroid receptor activator activity of SRA. The design of our primers for SRA as previously described (*3*), will detect all SRA isoforms containing core sequences. In the absence of data characterizing potential individual functions of SRA isoforms differing in the 5' and 3' regions outside of an intact core region, we assume that our measurement of all intact core SRA like RNAs correlates with the total SRA activity present in any one tissue sample. These primers, however, also detect a distinct but previously described isoform of SRA (Genbank accession number AA426601) containing a deletion of sequences within the SRA core region. Deletions within the core region were previously reported to result in loss of SRA activator function (*1*). Although the specific deletion found in the naturally occurring SRA deleted variant RNA has not been characterized per se, the currently published data suggest that it is likely that this deleted variant is inactive with respect to steroid receptor activator activity. It could therefore function to alter steroid signaling pathways in human breast tumors and may contribute to the more aggressive phenotype associated with poorer prognosis breast tumors, which include characteristics such as high grade and large tumor size.

In conclusion our data support a significant upregulation of total core SRA-like expression and likely activity during human breast tumorigenesis. This upregulation, in combination with that of other steroid receptor activators and increased expression of ERα are all likely to contribute to altered

estrogen sensitivity which occurs during and possibly contributes to human breast tumorigenesis.

Notes.

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Legends to Figures.

Figure 1.

Primers and expected PCR products for core SRA and deleted SRA. SRAcoreU and SRAcoreL primers anneal with SRA core sequences (white boxes, bases 28-720, see reference 1) and allow the amplification of a 662 bp and a 459 bp long fragment corresponding to the wild-type SRA core mRNA and a SRA mRNA deleted from bases 155 to 357 (SRAdel, see reference 3), respectively. The 5' and 3' variable regions of the different SRA isoforms previously identified (1) are indicated by gray boxes.

Figure 2.

Detection of SRA in normal breast tissue adjacent to matched primary, ER+ invasive breast cancer. RNA extracted from matched breast tumors and adjacent matched normal breast tissue was extracted from 19 different patients and assayed for SRA expression using RT-PCR as described in Materials and Methods. PCR products were separated on 6% acrylamide gels, which were dried, exposed to phosphor-imaging screens, and scanned using a Molecular Imager™-FX. **A.** A digitized image showing the results obtained from 4 sets of normal tissue (N) and matched tumor tissue (T) is shown. The arrows identify the expected 662 bp core SRA PCR product (SRA core, confirmed by sequence analysis) and a 459 bp deleted SRA variant PCR product (SRAdel), which was identified by sequence analysis to correspond to an SRA variant deleted in sequences from position 155 to 357 (Genbank accession number AF092038). **B.** Ethidium bromide stained gel of the RT-PCR analysis of *GAPDH* mRNA run in parallel for the same samples. The arrow identifies the expected 178 bp *GAPDH* PCR product.

Figure 3.

A. Comparison of the expression of SRA in adjacent normal breast tissue and matched primary breast tumors. For each patient (n = 19), SRA expression was quantified and expressed in arbitrary units corrected for *GAPDH* signal as described in the Materials and Methods. The results are presented as a scatter graph. The normal samples are represented by open squares and the tumor samples by filled squares. Each matched normal and tumor sample is joined by a line. The level of SRA expression in normal tissue is significantly different to the level of SRA expression in the tumor tissues (Wilcoxon matched pairs test, two-tailed, $p = 0.0004$).

B. Comparison of the relative expression of the deleted SRA variant in adjacent normal breast tissue and matched primary breast tumors. For each sample, the signal corresponding to the deleted SRA variant RNA (SRAdel) was measured using Quantity One™ software (Bio-Rad, Hercules, CA) and expressed as a percentage of the corresponding core SRA signal as described in the Materials and Methods. The results are presented as a scatter graph. The normal samples are represented by open squares and the tumor samples by filled squares. Each matched normal and tumor sample is joined by a line. There is no significant difference between the relative expression of SRAdel in normal samples and their matched adjacent tumors.

Figure 1.

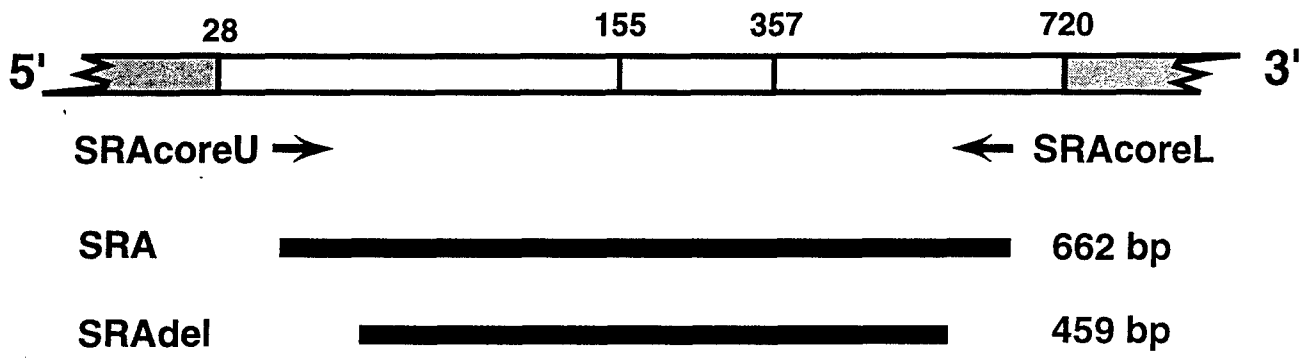


Figure 2

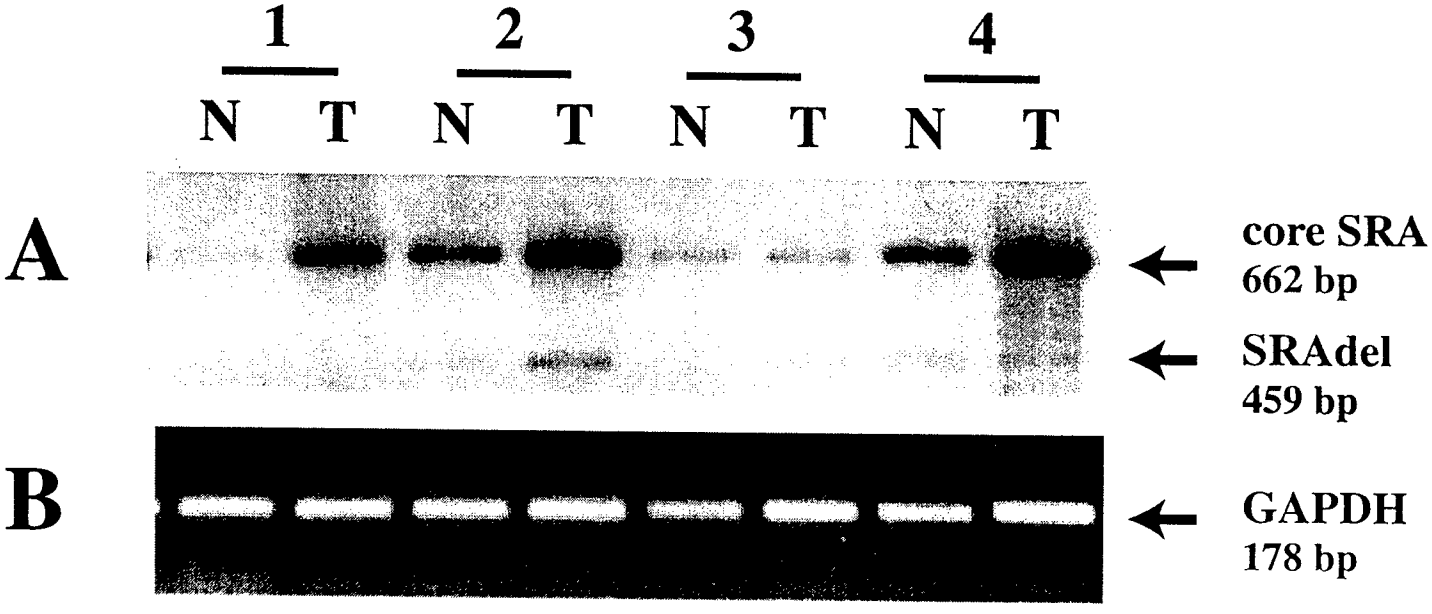
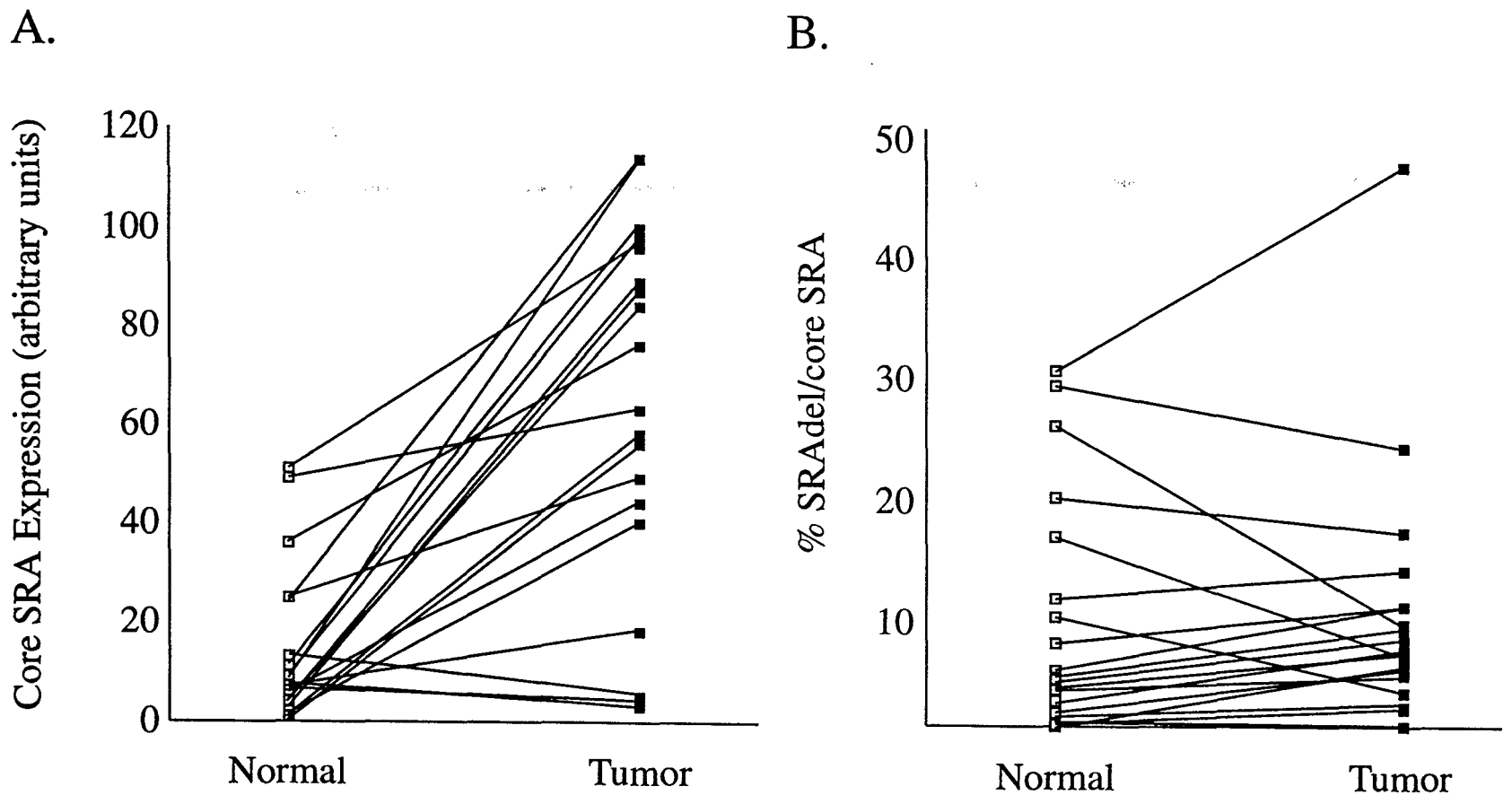


Figure 3


A.
Core SRA Expression (arbitrary units)
120
100
80
60
40
20
0
Normal
Tumor
B.
% SRAdel/core SRA
50
40
30
20
10
Normal
Tumor

APPENDIX 4

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Expression of a Repressor of Estrogen Receptor (ER) Activity (REA) in Human Breast Tissues. Simon SLR, Parkes A, Leygue E, Dotzlaw H, Watson PH*, Murphy LC. *Dept of Biochemistry & Medical Genetics; *Dept of Pathology, University of Manitoba, Winnipeg, Canada. R3E 0W3.*

ERα & some coactivators are upregulated during breast tumorigenesis. Recently, REA/BAP37 was identified as a repressor of ER. It was relevant therefore to determine if REA was expressed in breast tissue & if so was its expression altered during tumorigenesis. We measured REA mRNA levels in normal & neoplastic human breast tissues. A single ~1.7 kb REA mRNA was identified by Northern blot in breast cancer cells and biopsies. REA mRNA was measured by RT-PCR in ER+ breast tumors & matched adjacent normal tissue. Some breast tumors (12/19) contained higher levels of REA mRNA than their normal tissues, but this was not statistical significant, although SRA mRNA, an ER coactivator, was significantly increased in tumors vs matched normal tissue in this set (Wilcoxon, P=0.0004). REA mRNA levels varied amongst tumors and were found to be positively correlated (Spearman r=0.323, P=0.042) with ER level (determined by ligand binding) and inversely correlated with grade (Spearman r=-0.438, P = 0.0054). However, estrogen did not regulate REA mRNA expression in MCF7 cells. These data suggest that while REA expression may not be altered in early breast tumorigenesis, altered regulation may contribute to breast cancer progression.

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